Abstract

A gas chromatographic technique with flame ionization detection, which is based on a solid-phase extraction (SPE) procedure using mixed-mode SPE columns, for the simultaneous quantitation of dextropropoxyphene and norpropoxyphene in urine is presented. Urine is treated with sodium hydroxide in order to rearrange, by base catalysis, norpropoxyphene to norpropoxyphene amide, which is then extracted with these columns and chromatographed. The method is specific, linear over the range 0–2000 ng/mL, sensitive, and reproducible. The extracts are cleaner than those obtained with traditional liquid-liquid extraction procedure, which is an important feature in view of further mass spectrometric confirmation of narcotics and other drugs.

Introduction

Dextropropoxyphene (DPX) is an oral synthetic analgesic that is structurally related to methadone and was introduced to the United States market in 1957. It has been widely prescribed for the symptomatic relief of mild to moderate pain (1). This drug has been used in the treatment of heroin addicts (2); it has also been described as an abused drug since 1970 (3). Today, DPX continues to be widely abused in France, especially by heroin addicts (4), partly because the drug is easily available.

DPX is primarily metabolized via N-demethylation to a secondary amine, norpropoxyphene (NPX). Ring hydroxylation and ester hydrolysis are two additional pathways of DPX metabolism and may be followed by conjugation. In humans, N-demethylation is the major route of biotransformation of DPX. Pharmacokinetic data indicate that NPX has a longer elimination half-life (36 h) than the parent compound (12 h) (5).

Based on NPX toxicity in animals and on its resistance to naloxone, this metabolite is considered to be partially responsible for the toxic effects of DPX in humans, especially cardiotoxicity (5). Thus, its quantitative analysis in biological fluids is relevant in clinical toxicology.

Urine samples from drug addicts are often primarily screened for DPX by immunochromatographic techniques. Those allow for a qualitative analysis only. Moreover, the antibodies used in these methods generally have low affinity for NPX (6,7).

Different analytical methods for the determination of DPX and NPX have been published. These methods use high-performance liquid chromatography (HPLC) (8–12) or gas chromatography (GC) (7,13–20). Quantitation raises analytical problems: interlaboratory variation is high with coefficients of variation of approximately 45 and 65% for DPX and NPX, respectively (21). Care must be taken to avoid degradation under GC conditions (22). Moreover, most methods have incorporated the measurement of NPX after conversion into an amide, norpropoxyphene amide (NPXA), under strongly basic conditions to improve its chromatographic properties (13–18,20).

Liquid–liquid extraction was the standard procedure in the past. Nowadays, mixed-mode solid-phase extraction (SPE) is widely used for the screening of drugs in systematic toxicological analysis (23). These phases contain both hydrophobic and cation exchange functional groups to isolate various classes of drugs from biological matrices. The selectivity properties of this method allow for a better purification of urine extracts than liquid–liquid extraction. The advantages of SPE over liquid–liquid extraction also include lack of emulsion, more reproducible results, and reduced solvent use. Moreover, SPE technique can be automated. Up until now, few authors have proposed a SPE procedure for both DPX and NPX extraction (10,20).

Faced with an increasing rate of DPX poisoning in France (4) and given the necessity of confirm results referring to drug addicts by mass spectrometry (MS), we have developed a GC method for the simultaneous quantitation of this drug and its toxic metabolite in urine.

A liquid–liquid extraction procedure for both DPX and NPX was routinely used in our laboratory. Nevertheless, it was relevant to develop a standard compromise SPE procedure to isolate the largest number of drugs from biological matrices. The present paper describes an adaptation of the Varian cocaine and benzoylecgonine extraction procedure (24) for the simultaneous extraction of DPX and NPX.

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Materials and Methods

Materials

**Biological materials.** Urine samples from a normal population were obtained from the staff of our laboratory.

**Chemicals and reagents.** DPX hydrochloride was supplied by Houdé Laboratories (Puteaux, France); NPX maleate was obtained from Sigma (Lyon, France); and SKF 525-A (2-diphenylvalerate hydrochloride) was supplied by SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Stock solutions of DPX hydrochloride, NPX maleate, and SKF 525-A were prepared in methanol at a concentration of 100 mg/L and kept at -20°C. Methylene chloride, ethyl acetate, hexane (for trace analysis), methanol (for pesticide analysis), and isopropyl alcohol (HPLC grade) were purchased from SDS (Valdonna-Peypin, France). Acetonitrile (gradient grade) was from Merck (Darmstadt, Germany). Acetone, ethanol, butanol-1, ammonium hydroxide 28%, sodium hydroxide 35%, and hydrochloric acid 36% (RP Normapur) were purchased from Prolabo (Paris, France). The phosphate buffer (pH 6.0) was made up of 0.1 M potassium dihydrogen phosphate (Pro Analysi, Merck) dissolved in deionized water and adjusted to pH 6.0 with 1 M potassium hydroxide (Pro analysi, Merck).

**SPE apparatus.** Bond Elut Certify columns with a capacity of 10 mL, 150-mg sorbent phase were supplied by Varian Sample Preparation Products (Harbor City, CA). SPE columns were positioned on a Vac Elut Vacuum Manifold (Varian).

**Instrumentation.** The equipment used was a Varian Star 3400 CX gas chromatograph with a flame ionization detector (FID). Data acquisition and processing were achieved with the software Chrom-Card (Fisons Instruments, Arcueil, France). The column used was a fused-silica capillary column (DB-5 MS 5% phenyl silicone, 15-m length, 0.32-mm i.d., 0.25-µm film, J&W Scientific, Folsom, CA). The initial oven temperature of 100°C was increased at 15°C/min to reach a maximum temperature of 280°C and held for 5 min. The injector and detector temperatures were 250°C and 280°C, respectively. The injection mode was splitless (45 s). The carrier gas was helium (flow rate, 2 mL/min).

The identification confirmation of the nature of each compound detected in GC was carried out on a Fisons MD 800 mass spectrometer connected to a Fisons GC 8000. The mass spectrometer (MS) was operated in electronic impact mode (EI) at 70 eV and in positive chemical ionization mode (CI+) with methane as reagent gas. The temperature of the ion source was 200°C in EI and 150°C in CI+ mode. Injection and interface temperatures were 250°C and 280°C, respectively. A fused-silica capillary column (J&W DB-5 MS, 15 m x 0.25-mm i.d., 0.25-µm film thickness) was employed. The GC in splitless mode (45 s) operated isothermally at 100°C for 1 min, increased to 280°C at 10°C/min, and held for 5 min. The carrier gas was helium (flow rate, 1 mL/min). The GC-MS was monitored by Mass-Lab software (Fisons Instruments).

Methods

**Sample preparation.** Rearrangement by base catalysis of NPX to NPXA was the first step in our sample preparation. Seven samples were prepared at room temperature by spiking urine with DPX and NPX at concentrations of 0, 250, 500, 750, 1000, 1500, and 2000 ng/mL. One drop of 35% sodium hydroxide was added to each tube, and the samples were vortex mixed for 30 s. The pH of the samples was over 12; pH was further adjusted to pH 6 by the addition of HCl and 2 mL of phosphate buffer. SKF 525 (1000 ng/mL) was added as internal standard.

**Bond Elut Certify column preparation.** SPE columns were conditioned by passing 2 mL methanol and 2 mL phosphate buffer pH 6.0 slowly through the column successively. Precautions were taken to prevent column drying before sample application.

**Sample application.** The sample was poured into the cartridge reservoir and passed slowly through the sorbent bed.

**Column rinse.** The column was washed with 6 mL deionized water, dried under vacuum for 5 min, rinsed with 3 mL 0.1N HCl, dried under vacuum for 5 min, washed with 1 mL acetonitrile (rather than 9 mL methanol as in the cocaine and benzoylecgonine procedure), and dried under vacuum for 5 min.

**Elution.** DPX, NPX, and SKF 525-A were slowly eluted by 2 mL of methylene chloride–isopropyl alcohol (80:20) prepared daily with 2% ammonium hydroxide. The eluent was collected in a glass tube and slowly evaporated at room temperature.

**Injection.** The residue was reconstituted in 0.5 mL methylene chloride. The sample was transferred to a vial, slowly evaporated again under nitrogen at room temperature, and reconstituted in 50 µL ethyl acetate. One microliter of the solution was injected into the GC.

**Statistical analysis.** The Student's t-Test was used to compare the slopes of the regression lines of DPX and NPX extraction and to assess their correlation coefficients.

Results

**GC–FID and GC–MS analysis of DPX, NPX, and NPXA**

A typical GC–FID chromatogram of DPX, NPX, and SKF 525-A showed three peaks of low intensity for NPX when compared with internal standard and DPX (Figure 1).

When urine was extracted after the transformation of NPX to NPXA, the chromatogram showed a unique peak corresponding to NPXA (Figure 2).

**Adaptation of the cocaine and benzoylecgonine procedure to DPX and NPXA extraction procedure**

Seven urine samples spiked with DPX and NPX over the range of 0–2000 ng/mL were treated with a strong alkali hydroxide to form NPX and extracted following the cocaine and benzoylecgonine procedure. In this experiment, only DPX was recovered during GC; NPXA was not. Consequently, it was necessary to modify this procedure for the simultaneous extraction of DPX and NPXA.

**Determination of the fraction containing NPXA.** In order to understand why NPXA could not be recovered, all fractions of each step were collected to look for the presence of NPXA.

First, NPXA was not recovered in urine after passage through the SPE column, which indicated it was actually retained on
the sorbent. Then, rinse solvents were analyzed successively: deionized water, hydrochloric acid, and methanol. NPXA was recovered in the methanol fraction.

Choice of a rinse solvent. Several organic solvents were successively tested for their ability to clean the extracts without eluting NPXA. For this, methanol was replaced by 1 mL of another wash solvent: hexane, methylene chloride-isopropyl alcohol, butanol-1, ethyl acetate, ethanol, acetone, or acetonitrile. Only hexane and acetonitrile did not elute NPXA; acetonitrile was used as the wash solvent. Increasing the volume of acetonitrile from 1 mL to 2 mL did not improve the purity of extracts. Thus, 1 mL acetonitrile was used as the optimal wash condition in our SPE procedure.

Validation parameters

Linearity. The equations of linearity for DPX and NPXA, expressed as \( y = ax + b \), are given in Table I.

Limit of linearity. The limit of linearity was determined by extracting urine samples with increasing concentrations of DPX and NPX. The calibration curves of DPX and NPX were found to be linear over the range of 0–5000 ng/mL, with significant correlation coefficients \( r = 0.999 \) (\( p < 0.001 \)) and \( r = 0.996 \) (\( p < 0.001 \)), respectively.

Within- and between-day precision. Within-day precision was calculated from repeated analysis (\( n = 10 \)) during one working day by the same operator. Within-day precision was calculated for three concentrations of DPX and NPX. After conversion to NPXA, ten aliquots were extracted so that only the variations of the extraction recoveries were taken into account in the evaluation of the coefficient of variation. Results are given in Table II.

Between-day precision was calculated from the extraction of ten samples at the same concentrations of DPX and NPX. One extraction was performed per day (Table II).

Limit of detection. The limit of detection (LOD) was determined by adjusting the integration parameters of the software Chrom-Card (minimum area = 0, threshold = 0) to detect background peaks. Ten values of the ratio (background peak area)/(SKF 525-A peak area) were detected in the same period of the chromatogram near DPX and NPXA from ten independent blank urine extracts. The mean ratio value obtained was converted into a mean blank concentration \( B \) using the equations of the mean regression lines for DPX and NPXA. According to the expression \( LOD = 3B \), we found LODs of 27 and 48 ng/mL for DPX and NPX, respectively.

Limit of quantitation. According to the equation, the limit of quantitation (LOQ) = 10B, we found LOQs of 90 and 160 ng/mL for DPX and NPX, respectively.

These results were confirmed experimentally by performing a within-day precision study with 10 independent extracts at the

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**Figure 1.** GC–FID chromatogram of dextropropoxyphene, norpropoxyphene, and SKF 525-A at a concentration of 1000 ng/mL without transformation or extraction of norpropoxyphene to norpropoxyphene amide. Peak identification: 1, diphenylbutene isomer; 2, diphenylbutene isomer; 3, dextropropoxyphene; 4, SKF 525-A; 5, norpropoxyphene; 6, norpropoxyphene; 7, norpropoxyphene.

**Figure 2.** GC–FID chromatogram of dextropropoxyphene, norpropoxyphene, SKF 525-A at a concentration of 1000 ng/mL after transformation of norpropoxyphene to norpropoxyphene amide and SPE on spiked urine. Peak identification: 1, diphenylbutene isomer; 2, diphenylbutene isomer; 3, dextropropoxyphene; 4, SKF 525-A; 5, norpropoxyphene; 6, norpropoxyphene; 7, norpropoxyphene; 8, norpropoxyphene amide.
Table I. Linearity of the SPE Method

<table>
<thead>
<tr>
<th></th>
<th>DPX</th>
<th>NPXA</th>
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<tbody>
<tr>
<td>Linearity*</td>
<td>( y = 0.871 x + 9.25 \times 10^{-3} )</td>
<td>( y = 0.931 x - 1.40 \times 10^{-2} )</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>( r = 0.998 ) ( (p &lt; 0.001) )</td>
<td>( r = 0.998 ) ( (p &lt; 0.001) )</td>
</tr>
<tr>
<td>CV of the slope(^{\dagger})</td>
<td>3.30% ( (n = 10) )</td>
<td>4.03% ( (n = 10) )</td>
</tr>
<tr>
<td>Intercept(^{\ddagger})</td>
<td>0.52%</td>
<td>-0.76%</td>
</tr>
</tbody>
</table>

* The equations of linearity for DPX and NPXA are expressed as \( y = ax + b; x = \text{ratio (concentration of the analyte in the sample)/(concentration of the internal standard in the sample)}; y = \text{ratio (peak area of analyte)/(peak area of SKF 525-A)}; a = \text{mean value of 10 slope values}; b = \text{mean value of 10 intercept values}; a and b values were obtained from 10 regression lines over the range of 0-200 ng/mL established on the basis of one regression line per day.
\(^{\dagger}\) The coefficient of variation (CV) of the slope was defined as the ratio (standard deviation of n slope values/mean of n slope values) \times 100.
\(^{\ddagger}\) The intercept was expressed as a percentage of the value of the analytical response at a 100% analyte concentration.

Table II. Within-Day and Between-Day Precision

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>CV (within-day precision)</th>
<th>CV (between-day precision)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>5.25% 6.32%</td>
<td>7.09% 6.91%</td>
</tr>
<tr>
<td>750</td>
<td>3.71% 6.38%</td>
<td>5.61% 7.66%</td>
</tr>
<tr>
<td>1500</td>
<td>3.90% 4.36%</td>
<td>5.58% 6.02%</td>
</tr>
</tbody>
</table>

Table III. Retention Times for Dextropropoxyphene, Norpropoxyphene Amide, and Various Drugs Relative to SKF 525-A (Relative Retention Time)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecgonine methyl ester</td>
<td>0.317</td>
</tr>
<tr>
<td>MDA*</td>
<td>0.317</td>
</tr>
<tr>
<td>MDMA</td>
<td>0.364</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>not detected</td>
</tr>
<tr>
<td>EDDP</td>
<td>0.776</td>
</tr>
<tr>
<td>Methadone</td>
<td>0.870</td>
</tr>
<tr>
<td>DPX</td>
<td>0.905</td>
</tr>
<tr>
<td>SKF 525-A</td>
<td>1</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>1.02</td>
</tr>
<tr>
<td>Codeine</td>
<td>1.05</td>
</tr>
<tr>
<td>Codeethylne</td>
<td>1.08</td>
</tr>
<tr>
<td>Diazepam</td>
<td>1.09</td>
</tr>
<tr>
<td>Morphine</td>
<td>not detected</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>1.14</td>
</tr>
<tr>
<td>6-monoacetylmorphine</td>
<td>1.14</td>
</tr>
<tr>
<td>NPXA</td>
<td>1.16</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
<td>not detected</td>
</tr>
<tr>
<td>Methylyamphetamine</td>
<td>not detected</td>
</tr>
<tr>
<td>7-aminofluunitrazepem</td>
<td>1.22</td>
</tr>
<tr>
<td>Norbuprenorphine</td>
<td>not detected</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>not detected</td>
</tr>
</tbody>
</table>

* Abbreviations: MDA = methylenedioxyamphetamine, MDMA = methylenedioxyethylamphetamine, EDDP = ethylidene-2-dimethyl-1,5 diphenyl-3,1 pyrrolidine, DPX = dextropropoxyphene, NPXA = norpropoxyphene amide.

Extraction recovery. The extraction recovery of DPX was determined using SKF 525-A as the external standard. Extraction recovery, expressed as a percentage, was defined as the ratio (slope of the SPE regression line)/ (slope of the direct calibration curve). For DPX, these slopes were 0.802 and 0.931, respectively, giving an extraction recovery of 86.1%. Because NPXA was not available as a pure compound, calculation of the extraction recovery was not possible.

Interference. Normal components of six urine samples did not interfere with the analysis of DPX, SKF 525-A, or NPX. Eighteen retention times from narcotic or psychotropic drugs that can be found in the urine of drug addicts were investigated in order to exclude interferences with our method (Table III). None of these drugs interfered. A typical GC-FID chromatogram obtained with a patient's urine is presented in Figure 3.

Discussion

Chromatographic analysis

Chromatographic conditions. The necessity to further confirm the presence of narcotic drugs by mass spectrometry led us to use a GC procedure for the assay of DPX and NPX. The DB-5 MS J&W column, the injector split-splitless mode, and the column temperature program are classic conditions used in GC for the detection of narcotic drugs. The FID mode was found to be sensitive enough to detect molecules such as DPX and NPX, which were often present in high concentrations.

Stability of NPX. Typical direct FID chromatograms of NPX show a set of five peaks: peaks 1 and 2 are located at early retention times, whereas peaks 5, 6, and 7 are close together and found at later retention times (Figure 1).

In GC-MS, under methane chemical ionization conditions, the three corresponding mass spectra of peaks 5, 6, and 7 present a major ion at \( m/z \) 308. Furthermore, the presence of ions \( (M+ C_2H_5)^+ \) at \( m/z \) 336 and \( (M+ C_3H_7)^+ \) at \( m/z \) 348 is consistent with a compound of molecular weight 307 (Figure 4).

In EI mode, their mass spectra (Figure 5) were found to be identical to the spectrum of dehydrated NPXA (25). This corresponds to the previously determined mass of 307. These three peaks might be related to different isomers.

We checked the structure and nature of peaks 1 and 2, which corresponded to 2-diphenylbutene isomers and were also described as degradation products of DPX by Millard et al. (22). Therefore, NPX cannot be chromatographed directly.

However, if NPX is brought to pH 11 or above, it undergoes an intramolecular acyl shift leading to a stable molecule, NPXA. NPXA can be chromatographed without degradation and can be easily quantitated on the basis of a unique peak (peak 8, Figure 2).
Most of the GC methods described in the literature for the assay of DPX and NPX used this procedure to detect NPX (13–18,20).

In order to further validate the SPE method for DPX and NPX, it appeared necessary to test the linearity of the transformation of NPX to NPXA and the stability of NPXA and DPX. The formation of NPXA was proportional to the concentration of NPX over the range of 0–2000 ng/mL and exhibited a significant correlation coefficient ($r = 0.999$, $p < 0.001$).

NPXA stability at pH 6 was verified; the slope values of recovery of NPXA extracted at pH 12 or 6 were not significantly different in either series ($p > 0.1$). Therefore, NPXA recovery was not affected by pH change, allowing for its extraction with the SPE method.

This reaction was not influenced by incubation time in alkaline solution or by temperature in our experimental conditions. The slope values of NPXA recovery with an incubation time of 60 min at room temperature or 60 min at $60^\circ$C, both at pH 12, were not significantly different from 30 s at room temperature ($p > 0.2$ and $p > 0.8$). These parameters were kept as the simplest transformation condition of NPX to NPXA.

Moreover, GC–MS analysis allowed us to verify the complete transformation of NPX to NPXA.

**Stability of DPX.** Under our experimental procedure, we have shown that DPX was not affected by strong alkali treatment; the slope values of DPX recovery after extraction performed at pH 6 before or after conversion of NPX to NPXA were not significantly different ($p > 0.1$), thus allowing for the simultaneous extraction and quantitation of DPX and NPX.

However, the degradation of DPX during GC described by Millard et al. (22) led us to test thermal decomposition of DPX as related to injection temperature.

No degradation was observed under the injection temperature of $200^\circ$C. Between $210^\circ$C and $250^\circ$C, only diphenylbutene isomers were detected as degradation products, corresponding to a maximum loss of 7% DPX. Above $260^\circ$C, a second degradation mechanism occurred, which led to the loss of propionic acid.

Finally, in order to get a satisfactory volatilization of NPXA and to minimize injector pollution, a compromise temperature of $250^\circ$C was chosen to obtain an optimal analysis.

**Choice of internal standard.** SKF 525-A, a classical internal standard used by several authors (7,13,17), was chosen in our method for its tertiary amine structure related to DPX and for its ability to be extracted in the same conditions as DPX. As can be seen in Figures 1 and 2, SKF 525-A (peak 4) emerged from DPX and NPXA well-separated.

**Validation parameters.** The SPE method was found to be linear over a wide range (0–2000 ng/mL) with a correlation coefficient of 0.998 for both DPX and NPXA. The value of the intercept of the regression line can be expressed as a percentage of the value of the analytical response at a 100% analyte concentration. This percentage should normally be within the range of –2.0 to 2.0% to consider the validation satisfactory (26). These values were found to be 0.52% and –0.76% for DPX and NPXA, respectively.

Considering the within-day and between-day validation parameters, the method was found to be reproducible. The LOQs of DPX (90 ng/mL) and NPX (160 ng/mL) were sensitive enough and were valuable for monitoring drugs in toxicology, where high levels of DPX and especially NPX are frequently detected in urine. The extraction recovery of DPX was significantly higher in SPE (86.1%) than in our liquid–liquid extraction method (78.5%). For NPX, extraction was more efficient in SPE than in liquid–liquid extraction.

**Conclusion**

An SPE method was developed for the simultaneous extraction of DPX and NPX after the transformation of NPX to NPXA in human urine. This method was found to be rapid and convenient. It could be automated and extended to other biological fluids. The extracts are cleaner than those obtained with the traditional liquid–liquid extraction procedure.
Figure 4. GC-MS chromatogram and spectra of norpropoxyphene registered in positive chemical ionization (CI+*) mode with methane as the reagent gas.
Figure 5. GC–MS chromatograms and spectra of norpropoxyphene registered in positive chemical ionization (CI§) and electron impact (El) modes.
The chromatographic conditions were optimized in order to avoid degradation of DPX and NPX and to obtain linear results over a wide range of concentrations. The method is sensitive enough and provides good within- and between-day precision parameters.

Toxicology laboratories are increasingly faced with addiction trends that often include DPX; in such cases, the proposed method can be very useful in confirming immunochemical urinary screening. It must be remembered that DPX is not recognized by antiantiopiate antibodies and that anti-DPX antibodies have low affinity for NPX, which is only sometimes present in the urine samples because of its longer elimination halftime. Moreover, European toxicological quality controls require DPX and NPX quantitation in urine.

Lastly, it is relevant to use a specific and reliable method in forensic analysis that is compatible with GC-MS confirmation. Nevertheless, in order to correlate biological and clinical data for a diagnosis, blood concentration determination is necessary. Validation of this method in blood matrix is our present aim.

References

6. EMIT d.a.u. Propoxyphene assay, package insert, Syva Co.

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